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Synthetic Color Detection by HPLC (High Performance Liquid Chromatography) or Ultra HPLC (UHPLC)

Scope and Application

This method will be used to detect the presence of synthetic colors in alcohol beverage products using a High Performance Liquid Chromatography-Diode Array Detector (HPLC-DAD) system or an Ultra High Performance Liquid Chromatography-Photo Diode Array (UPLC-PDA) system. Many alcohol beverage products contain synthetic coloring agents. As per 21 CFR 74, there are seven FDA Certified Colors for use in beverage products: FD&C Blue #1, FD&C Blue #2, FD&C Red #3, FD&C Red #40, FD&C Yellow #5, FD&C Yellow #6, and FD&C Green #3. FD&C Yellow #5 must be specifically declared on the beverage label (21 CFR 74.705). This method can be used to detect these seven colors. Additionally, this method can be used to detect synthetic colors that are not allowed in the United States or that are only certified for use in drugs and/or cosmetics (please refer to the method validation package Colors Library logbook). This method is applicable to beverage alcohol products.

Regulatory Tolerances:

As per 21 CFR 74, there are seven FDA Certified Colors for use in beverage products: FD&C Blue #1, FD&C Blue #2, FD&C Red #3, FD&C Red #40, FD&C Yellow #5, FD&C Yellow #6, and FD&C Green #3.

As per 21 CFR 74.705, FD&C Yellow #5 must be specifically declared on the beverage label.

Levels and Limitations

This method is based on the unique retention times and spectra of synthetic colors when analyzed by HPLC-DAD or UHPLC-PDA. The retention times and spectra obtained for samples are compared to the retention times and spectra obtained for standards as a means of identifying the presence of synthetic color in the samples.

This method is intended to yield a qualitative determination of the presence or absence of a synthetic coloring agent. During validation, the detection limit of this method was estimated to be approximately 1 ppm in matrix.

This method is applicable to alcohol beverage products.

Supplemental Documents

NLC:WG:305 - Agilent 1290 Startup and Storage User Guide

Instrument specific manual(s), as available

Equipment

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Instrumentation and Run Conditions:

HPLC: Agilent 1290 Infinity High Performance Liquid Chromatography system, or equivalent
Agilent diode array detector G4212A, or equivalent
Agilent autosampler G4226A, or equivalent
Agilent temperature control compartment G1316C, or equivalent
Agilent quaternary pump G4204A, or equivalent

Wavelengths: Scan across the entire range (190-640nm). Additional wavelengths may be individually selected based on the anticipated need. For example:
254 nm general screen for all colors
625 nm for FD&C Blue #1
605 nm for FD&C Blue #2
500 nm for FD&C Red #40
540 nm for FD&C Red #3
425 nm for FD&C Yellow #5
480 nm for FD&C Yellow #6
625 nm for FD&C Green #3

Injection Volume: 20 µL

HPLC Column Temperature: 35°C

HPLC Flow Rate: 2.0 mL/min

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Mobile Phases: A: Buffer (0.015 M potassium phosphate monobasic)
***ALTERNATE MOBILE PHASE A:** 10% Methanol:90% Buffer
B: Methanol

HPLC Gradient:
0.00 min: 90% A, 10% B
3.00 min: 90% A, 10% B
18.00 min: 10% A, 90% B

Run Time: 18.00 minutes with 10.00 minutes post-run time with 90% A, 10% B

Degasser: Optional

Software: OpenLAB CDS Chemstation Edition for LC & LC/MS Systems, Rev. C.01.07 [27] or equivalent

Guard and Column:
*Prodigy ODS 4 x 3.0 mm, AJO-4287, or equivalent
*Prodigy ODS 3, 100Å, 00F-4097-E0, 150 x 4.6 mm, 5 µm, or equivalent

***NOTE:** As this is a qualitative method, there are many potentially acceptable C18 columns and guard columns. If a column is demonstrated to be capable of separating the seven certified colors in a 10% ethanol solution in a repeatable fashion, with the spectra matching those found in this method, the column and guard column may be deemed equivalent. If a peak elutes at the transition from isocratic to gradient, the chromatography will be negatively affected. When this is the case, use **ALTERNATE MOBILE PHASE A** so that the peak will elute before the change to gradient.

OR

UHPLC: Waters Acquity Ultra Performance Liquid Chromatography System, or equivalent
Binary Solvent Manager PN: 186015001, or equivalent
Sample Manager PN: 186015006, or equivalent
High Temperature Column Heater PN: 186015010, or equivalent
PDA eλ Detector PN: 186015033, or equivalent

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Wavelengths:	Scan across the entire range (190-650nm). Additional wavelengths may be individually selected based on the anticipated need. For example: 254 nm general screen for all colors 625 nm for FD&C Blue #1 605 nm for FD&C Blue #2 500 nm for FD&C Red #40 540 nm for FD&C Red #3 425 nm for FD&C Yellow #5 480 nm for FD&C Yellow #6 625 nm for FD&C Green #3
Injection Volume:	2 µL
Column Temperature:	35°C
Flow Rate:	0.5 mL/min
Mobile Phases	A: Buffer (0.015 M potassium phosphate monobasic) ***ALTERNATE MOBILE PHASE A:** 10% Methanol:90% Buffer B: Methanol
UHPLC Gradient:	0.00 min: 95% A, 5% B 1.00 min: 95% A, 5% B 6.00 min: 20% A, 80% B
Run Time:	6 min with 3 min post-run at 95% A, 5% B
Software:	Empower Pro Empower 2 Software, or equivalent
UHPLC Guard and Column:	*Phenomenex SecurityGuard™ ULTRA C18 cartridge PN: AJ0-8768 connected to SecurityGuard™ ULTRA Holder PN: AJ0-9000 *Phenomenex Kinetex 100Å, PN: 00B-4475-AN 50 x 2.10 mm, or equivalent

***NOTE:** As this is a qualitative method, there are many potentially acceptable C18 columns and guard columns. If a column is demonstrated to be capable of separating the seven certified colors in a 10% ethanol solution in a repeatable fashion, with the spectra matching those found in this method, the column and guard column may be deemed equivalent. If a peak elutes at the transition from isocratic to gradient, the chromatography will be negatively affected. When this is the case, use **ALTERNATE MOBILE PHASE A** so that the peak will elute before the change to gradient.

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Analytical balance Sartorius 1602 MP8-1, or equivalent

For crème liquors only:
Shaker, Glas-Col ® Multi-Pulse Vortexer model 099A VB412FP, or equivalent
Centrifuge, Sorvall Legend RT+, or equivalent

Glassware and Supplies:

2 mL autosampler vials (preferably amber) and caps, National Scientific C4012-1 vials and C4011-1AHP caps, or equivalent
Class A volumetric flasks, 200 mL and 1 L, or as needed
Graduated cylinders, 100 mL and 1 L, or as needed
Weighing funnels (if desired), Tradewinds Direct DPWFPP1S, or equivalent
Buffered mobile phase filters (if desired), Millipore PTFE 0.45µm, Fisher SJLHM4710, or equivalent
For crème liqueurs only: Ethanol-resistant centrifuge tubes with caps, 50 or 100mL size. For non-dairy crème liqueurs glass tubes are required.
SPE-NH_2 cartridges (for crème liqueurs and for non-crème liqueurs, as needed), Varian part number 12113014, or equivalent (larger or smaller cartridges with more or less packing material are acceptable, as the method is qualitative)

Reagent and Sample Preparation and Handling

Reagents: (Specific vendors and product numbers are listed for convenience. Equivalent products may be used.)

Deionized (DI) water, 18 megaohm or better
Ethyl Alcohol, 200 proof, Pharmco 111000200
1 N Sulfuric Acid (for SPE only), Fisher certified, SA212-1
Potassium Phosphate Monobasic, ACS reagent ≥99.0%, Fisher P285-500
Methanol, HPLC gradient grade ≥99.8%, Sigma 34885-4x4L
Synthetic colors (purity may vary, as the method is qualitative)
- FD&C Blue #1, 94%, Sensient Food Colors 05601
- FD&C Blue #2, 91%, Sensient Food Colors 05602
- FD&C Yellow #5, 90%, Sensient Food Colors 08005
- FD&C Yellow #6, 91%, Sensient Food Colors 08006
- FD&C Red #3, 89%, Sensient Food Colors 07003
- FD&C Red #40, 90%, Sensient Food Colors 07700
- FD&C Green #3, 87%, Sensient Food Colors 06503
- Additional colors as needed (or as available)

Methylene Chloride (for non-dairy crème liqueurs only), Optima grade, Fisher D151-4

Preparation of Solutions:

The following solutions are needed in this method [**Note:** weights and volumes may be adjusted as needed; however, ensure that the ratios remain the same]:

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A. Mobile Phase A Buffer: 0.015 M potassium phosphate monobasic (KH_2PO_4). For example, weigh 2.1 (±0.05) g KH_2PO_4, dissolve in and dilute to 1 L with DI water. Filtering buffer prior to use is recommended with at least a 0.45 µm filter. If possible, the buffer should be stored in the refrigerator when not in use and should be stable for three months.

B. **ALTERNATE MOBILE PHASE A:** Prepare Mobile Phase A Buffer as above, then prepare a mixture containing 90% Mobile Phase A and 10% methanol. Please refer to the Instrument and Run Conditions section for information about when this mobile phase should be used.

C. 10% Ethanol: Measure 100 mL ethanol and 900 mL DI water. Mix well and allow to equilibrate before use. This solution is stable for two years when stored at room temperature.

D. 50:50 ethanol:1N sulfuric acid (if needed for SPE): Measure 50 mL ethanol and mix with 50 mL 1 N sulfuric acid. This solution is stable for two years when kept sealed in an amber bottle at room temperature.

Preparation of Working Standards:

Working standards are solutions of approximately 25 ppm synthetic color in 10% ethanol solution. Prepare working standard solutions as follows:

A. For example, weigh (or weigh and transfer) 0.0050 (± 0.005) g of each neat standard into individual 200 mL volumetric flasks [**Note**: As this is a qualitative method, adjusting for purity is not necessary.]

B. Bring each stock to volume with 10% ethanol solution, and invert to dissolve and mix. Store in a sealed container and protect from light (for example, store in amber bottles or store clear containers inside a cabinet). When protected from light, standard solutions are stable for two years when stored at room temperature.

C. Place an aliquot of each standard solution into separate autosampler vials for analysis or prepare mixtures of standards, as necessary. (Preferably, use amber vials or an autosampler that is capable of protecting from light; however, the standard solutions are stable when exposed to light for at least one week).

Preparation of Laboratory Control Sample (LCS):

There is no laboratory control sample in this method, as it is qualitative.

Preparation of Samples (non-crème liqueurs):

All non-crème liqueurs should be filtered using a 0.2µm filter in to an autosampler vial for analysis.

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[**Note**: Solid Phase Extraction (SPE) may be helpful in some cases. In the event of extremely low concentrations of added colors, a co-eluting peak, or other matrix interference, the following procedure should be used:

1. Rinse the SPE cartridge with one column volume of ethanol. When there is approximately 0.25" of ethanol remaining, add one column volume of DI water.
2. When there is approximately 0.5" of water remaining, transfer approximately 50 mL sample through a SPE-NH_2 cartridge connected to a manifold with a vacuum of -2" Hg. If the sample contains high solids, dilute with DI water as needed.
3. Wash SPE cartridge with 10 mL ethanol and then 10 mL water.
4. When all water has been removed from SPE cartridge, allow to dry under vacuum for 5 minutes.
5. Slowly add 1 to 3 mL 50:50 ethanol:1N sulfuric acid to the SPE cartridge. Observe the color band travel down the packing material and when the colored liquid is observed at the tip, begin collection of ~2 mL of sample and transfer to an autosampler vial for analysis. (Note: the more packing material in the SPE the more eluent will be required to rinse out the colors). The eluent may be diluted with 10% ethanol, if desired.

Extraction of Samples (dairy crème liqueurs):

Crème liqueurs have a special matrix with high fat content. The extraction procedure for this type of product should be as follows:

1. Transfer 25 mL sample to a 100 mL screw cap centrifuge tube. Add 25 mL ethanol, cap, and shake vigorously for two minutes.
2. Centrifuge at 2500 rpm for ten minutes
3. Rinse the SPE cartridge with one column volume of ethanol. When there is approximately 0.25" of ethanol remaining, add one column volume of DI water.
4. When there is approximately 0.5" of water remaining, pass supernatant layer of centrifuged sample through a SPE-NH_2 cartridge (Varian part number 12113014, or equivalent) connected to a manifold with a vacuum of -5" Hg.
5. Repeat steps 1-3 so that a total of 50 mL of sample is delivered onto the SPE cartridge.
6. Wash SPE cartridge with 10 mL ethanol and then 10 mL water.
7. When all water has been removed from SPE cartridge, allow to dry under vacuum for 5 minutes.
8. Slowly add 1 to 3 mL 50:50 ethanol:1N sulfuric acid to the SPE cartridge. Observe the color band travel down the packing material and when the colored liquid is observed at the tip, begin collection of ~2 mL of sample and transfer to an autosampler vial for analysis. (Note: the more packing material in the SPE the more eluent will be required to rinse out the colors). The eluent may be diluted with 10% ethanol, if desired.

Extraction of Samples (non-dairy crème liqueurs):

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Crème liqueurs have a special matrix with high fat content. The extraction procedure for this type of product should be as follows:

1. Transfer 25 mL sample to a 100 mL screw cap centrifuge tube. Add 25 mL ethanol, cap, and shake vigorously for one minute. Then add 25 mL methylene chloride, cap and shake vigorously for two minutes.
2. Centrifuge at 2500 rpm for ten minutes
3. Rinse the SPE cartridge with one column volume of ethanol. When there is approximately 0.25” of ethanol remaining, add one column volume of DI water.
4. When there is approximately 0.5” of water remaining, pass supernatant layer of centrifuged sample through a SPE-NH_2 cartridge (Varian part number 12113014, or equivalent) connected to a manifold with a vacuum of -5” Hg.
5. Repeat steps 1-3 so that a total of 50 mL of sample is delivered onto the SPE cartridge.
6. Wash SPE cartridge with 10 mL ethanol and then 10 mL water.
7. When all water has been removed from SPE cartridge, allow to dry under vacuum for 5 minutes.
8. Adjust vacuum to -2” Hg. Collect the eluent from 1 to 3 mL 50:50 ethanol:1N sulfuric acid through the SPE and transfer to an autosampler vial for analysis. (Note: the more packing material in the SPE the more eluent will be required to rinse out the colors). The eluent may be diluted with 10% ethanol, if desired.

Procedures

The suggested order of the sequence should be:

1. 10% ethanol blank to ensure no trace of synthetic color on the column.
2. One or two injections of a single or mixed standard solution (depending on instrumentation used). If these injection(s) satisfy Quality Control #2, these injection(s) may be used as the standards for the run.
3. Single injections of standard solutions, as necessary. For example, standards of additional synthetic colors when the sample paperwork indicates that a synthetic color other than the seven certified colors has been added to a product.
4. Single injections of sample solutions.
5. Additional injections of standard solutions, as needed. For example, if a sample appears to have a synthetic color from the color library that was not yet injected, run the suspected color standard.
6. 10% ethanol blank to ensure no trace of synthetic color is left on the column.

Note: If the peak intensity of a color is large, it is recommended that the sample be diluted as necessary.

Quality Control

1. The 10% blank injections should not contain any traces of the seven certified synthetic colors. Flush or regenerate column according to column instructions if needed. Replace guard column if needed. Replace column if needed.

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2. The retention times of the synthetic color standard solutions should be consistent with historical data, as well as the duplicate injection of standard solution, if a duplicate was run. Retention times may shift depending on the condition of the column, the pressure of the overall HPLC/UHPLC system, or due to the injection of an undiluted SPE extract. If the retention times are unsatisfactory, explore traditional HPLC/UHPLC troubleshooting techniques (e.g., ensure that the system is not leaking at any connections, when pressure is lower than normal). If an SPE extraction was used, the analyst may opt to prepare the All 7 Certified Colors sample using the SPE method.

3. As the method is qualitative, there is no LCS or standard curve to evaluate.

4. Each new lot of SPE cartridges are to undergo QC testing by preparing both a 10% ethanol blank sample as well as an All 7 Certified Colors sample using the SPE sample preparation method. The blank sample should contain no synthetic colors while the All 7 Certified Colors sample should contain all the 7 certified colors and no others. If these two QC samples pass, then the lot of SPE cartridges can be used for sample preparation.

Calculations

As the method is qualitative, there are no calculations.

Reporting Results

This method is based on the unique spectra of synthetic colors when analyzed by HPLC-DAD or UHPLC-PDA. The spectra obtained for samples are compared to the spectra obtained for standards as a means of identifying the presence of synthetic color in the samples. **Figure 1** through **Figure 7** show the spectra for the seven certified colors. **Figure 8** and **Figure 9** show an example chromatogram of a mixture of all seven standard solutions at 254 nm using an HPLC and UHPLC, respectively.
Please refer to these spectra, the method logbook, as well as the Instrumentation and Run Conditions section for wavelengths of interest for particular synthetic colors.

Results are reported as either “detected” or “not detected” for each appropriate synthetic color, as per lab policy or current LIMS requirements. A chromatogram showing the synthetic color peaks eluting in time plus the individual color spectra across all collected wavelengths for both the samples and the applicable standards should be included in the raw data package for each sample.

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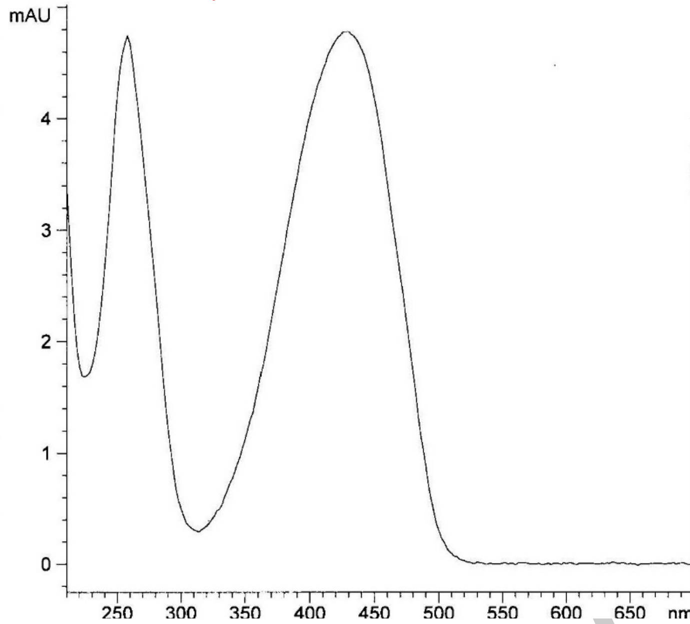

Figure 1. FD&C Yellow #5

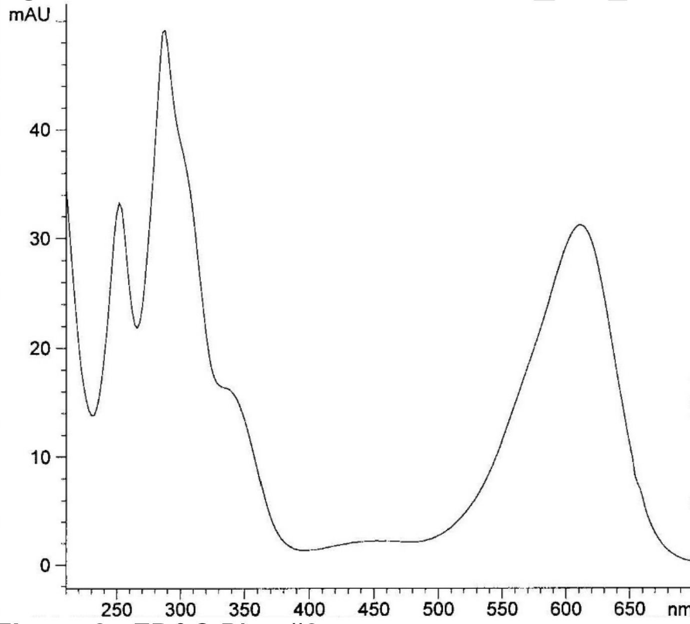

Figure 2. FD&C Blue #2

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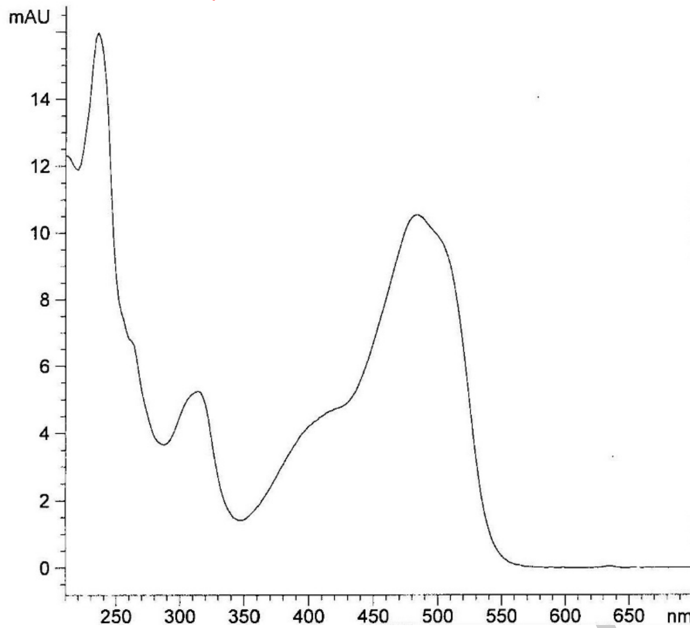

Figure 3. FD&C Yellow # 6

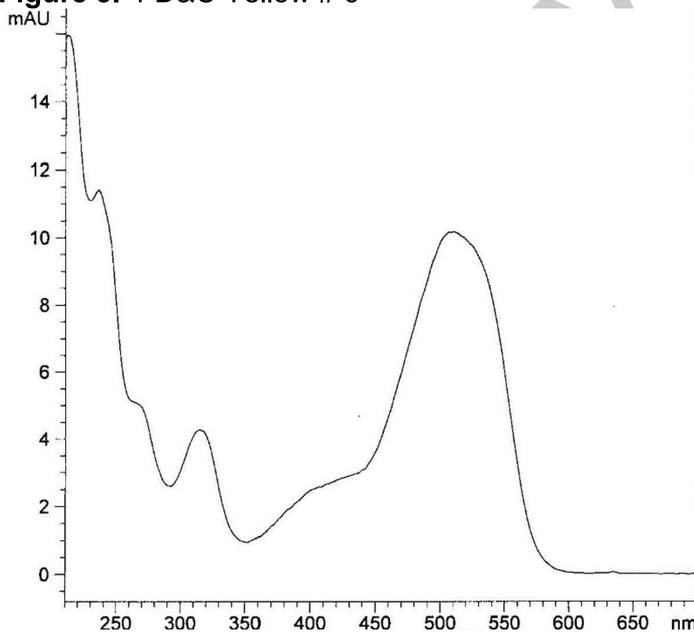

Figure 4. FD&C Red #40

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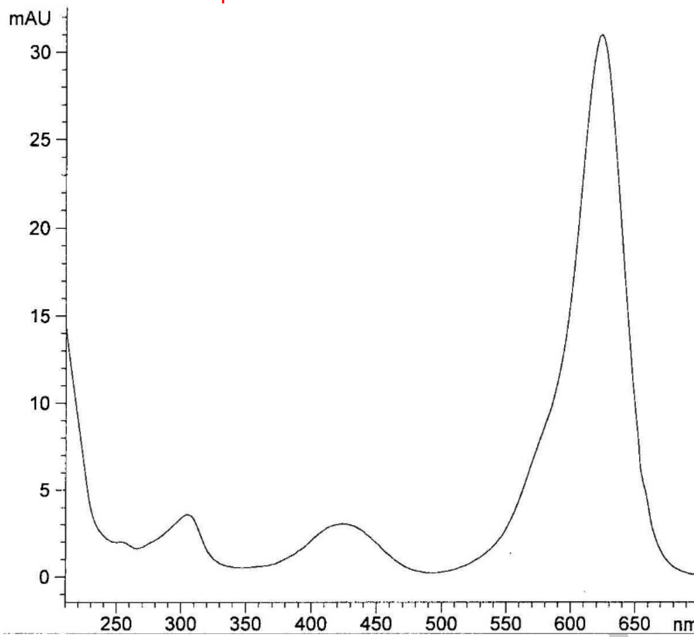

Figure 5. FD&C Green #3

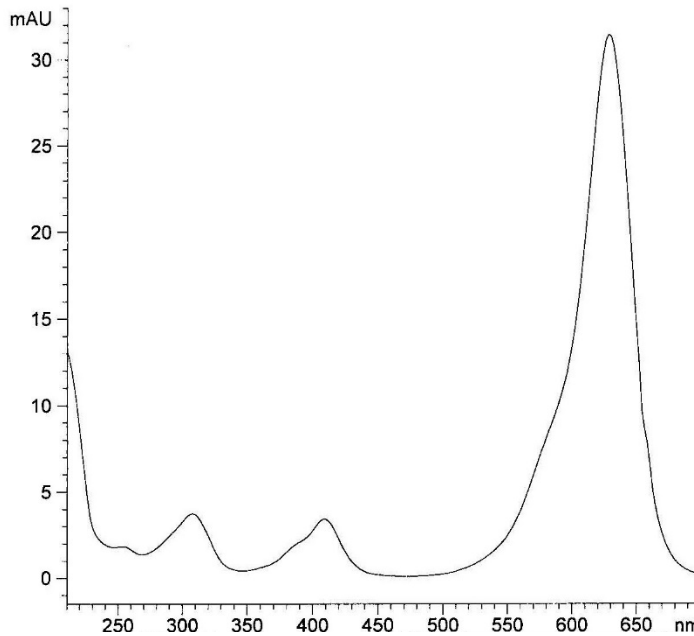

Figure 6. FD&C Blue #1

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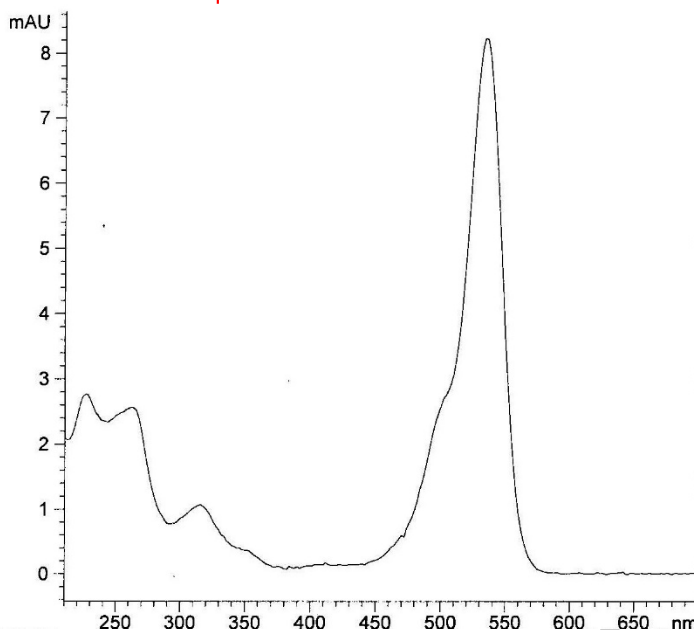

Figure 7. FD&C Red #3

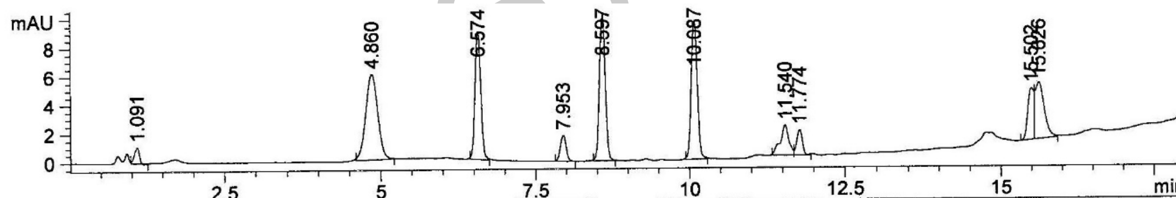

Figure 8. Chromatogram of a mixture of all seven standard solutions, 254 nm, using an HPLC. FD&C Yellow #5 is the peak at 4.860 minutes. FD&C Blue #2 is the peak at 6.574 minutes. FD&C Yellow #6 is the peak at 8.597 minutes. FD&C Red #40 is the peak at 10.087 minutes. FD&C Green #3 is the peak at 11.540 minutes. FD&C Blue #1 is the peak at 11.774 minutes. FD&C Red #3 is the peak at 15.626 minutes.

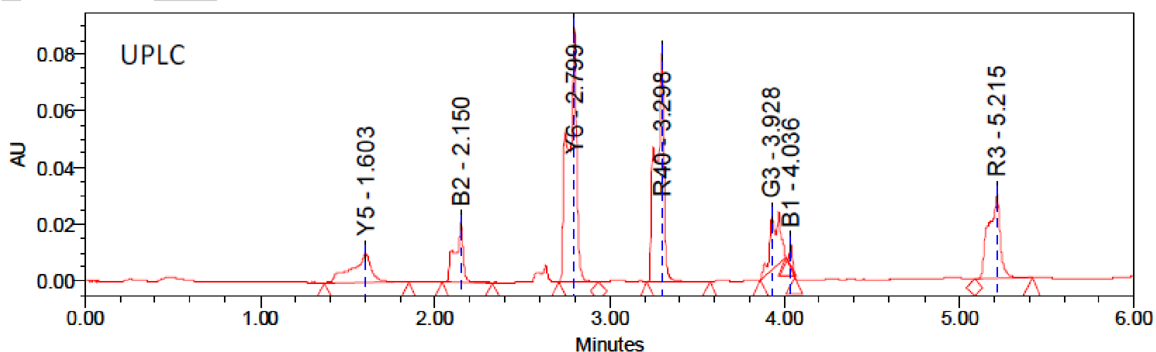

Figure 9. Chromatogram of a mixture of all seven standard solutions, 254 nm, using a UPLC. FD&C Yellow #5 is the peak at 1.603 minutes. FD&C Blue #2 is the peak at 2.150 minutes. FD&C Yellow #6 is the peak at 2.799 minutes. FD&C Red #40 is the

peak at 3.298 minutes. FD&C Green #3 is the peak at 3.928 minutes. FD&C Blue #1 is the peak at 4.036 minutes. FD&C Red #3 is the peak at 5.215 minutes.

Safety Notes

Normal laboratory safety protocol should be followed. Personnel should follow good laboratory practices such as wearing protective eye wear, gloves, and a lab coat.

Consult the MSDS for any chemicals used that are unfamiliar. All chemicals shall be considered hazardous - avoid direct physical contact.

High proof alcohol products are flammable. Ethanol burns with an almost invisible blue flame.

If methylene chloride is used, be aware of the following: according to OSHA Standard Number 1910.1052, "Employees exposed to methylene chloride are at increased risk of developing cancer, adverse effects on the heart, central nervous system and liver, and skin or eye irritation. Exposure may occur through inhalation, by absorption through the skin, or through contact with the skin." If desired and sufficient advanced notice given, methylene chloride exposure monitors can be obtained from the Safety Coordinator.

References

Martin et al., J. Associ. Off. Anal. Chem., vol. 61, No. 4, 1978, pp. 908-910.

Code of Federal Regulations (2008) Title 21 Part 74. *U.S. Government Printing Office, Washington, DC 20402-001.*

Dugar et al., Journal or AOAC International, vol. 77, No. 5, 1994.

Location of Validation Package

Quality System Files

Required Training, Certification and Re-certification.

1. In-house training by a certified chemist in HPLC or UHPLC operation.

2. Periodically, chemists are re-tested for competency (e.g., every 5 years) and/or given proficiency testing.

Revision History

Rev. 1 – initial revision
Rev. 2 – Extensive edits.

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